PHYSIOLOGY OF INSECT DIAPAUSE. XII. THE MECHANISM OF CARBON MONOXIDE–SENSITIVITY AND –INSENSITIVITY DURING THE PUPAL DIAPAUSE OF THE CECROPIA SILKWORM[1]

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In the preceding paper of this series (Harvey and Williams, 1958), the effects of cyanide on the heartbeat of the Cecropia silkworm were studied during successive stages in metamorphosis. When suitable allowance was made for the anaerobic capacity of the diapausing pupa, the heartbeat at all stages was found to be sensitive to cyanide.

This finding, in itself, suggests that the heartbeat throughout the life history depends on the function of cytochrome oxidase. However, it will be recalled that cyanide combines with a number of enzymes in addition to cytochrome oxidase (Warburg, 1949). For this reason we have re-examined the matter making use of a far more specific inhibitor, carbon monoxide. In animals lacking hemoglobin, a light-reversible inhibition by carbon monoxide is sufficient proof of the presence and functional activity of cytochrome oxidase (Hill and Hartree, 1953).

Materials and Methods

1. *Experimental animals*

Diapausing pupae and adult moths of the giant silkworm, *Platysamia cecropia* L., were used as experimental animals. The diapausing individuals were of two types: pupae stored at 25° C. and utilized within three months after the pupal molt; and pupae stabilized in a permanent diapause by removal of the brain at least one month prior to use (Williams, 1946). In a number of cases, parallel experiments were performed on the related giant silkworm, *Telea polyphemus* Cram.

2. *Observations of hearts*

Due to surface reflection from the pupal cuticle, the heartbeat is not visible in the intact insect. Hearts were therefore studied after isolation as previously described (Harvey and Williams, 1958). Subsequently, in collaboration with Dr. Ned Feder, we found that the heart becomes plainly visible and can be studied in the intact pupa under the following circumstance. The light source is equipped with a polarizing filter. A second polarizing filter is placed below the objective of the dissecting microscope. When the planes of polarization are "crossed," surface

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reflection is totally eliminated. Except in very darkly pigmented individuals one can then clearly observe the beating heart.

3. *Exposure of hearts to gases at elevated pressures*

A series of from four to six hearts was isolated and pinned to an elongate paraffin-coated tray of Lucite. Ringer's solution (Ephrussi and Beadle, 1936), containing phenylthiourea and streptomycin sulfate, was then added until each heart was covered by a film of the solution. In experiments with intact pupae the insects were placed dorsal side up on a Lucite tray equipped with plastic cradles to accommodate the individual pupae.

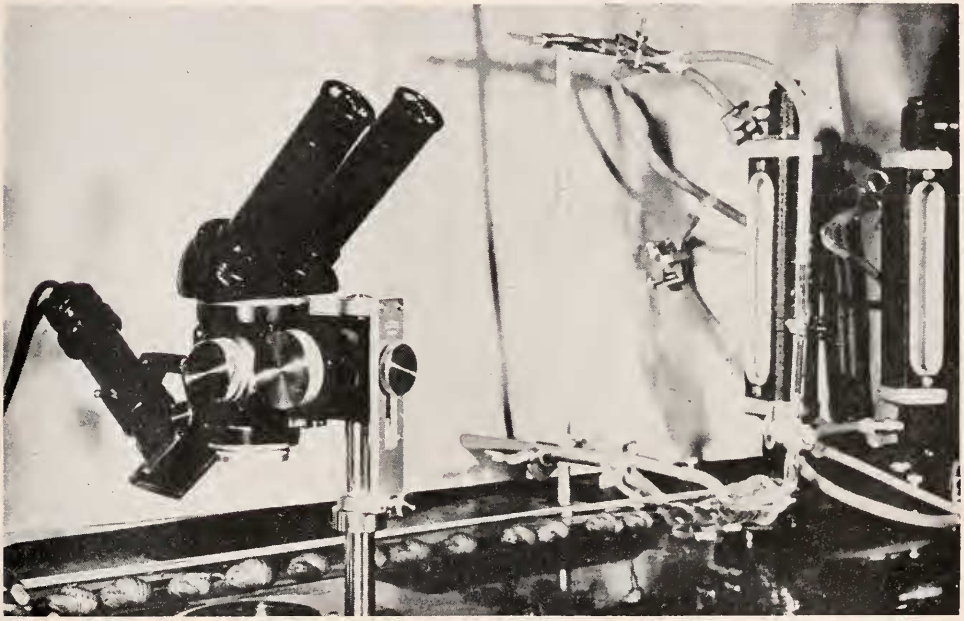


FIGURE 1. Apparatus for the exposure of pupae or of isolated hearts to flowing gas mixtures.

The tray with either isolated hearts or intact pupae was then inserted into a 3.5-liter Lucite chamber constructed to withstand high pressure (Schneiderman and Feder, 1954). The air-filled chamber was then sealed and compressed with carbon monoxide, the positive pressure being read from a gauge calibrated in pounds per square inch. The oxygen tension was therefore that of the initial air-filled chamber (21% atm.). In experiments utilizing oxygen pressures less than 21% atm., the chamber was first flushed with ten volumes of carbon monoxide. The system was then compressed with oxygen to a pre-determined pressure, making use of a mercury manometer. Finally, the chamber was further compressed with carbon monoxide.

4. *Exposure of hearts to flowing mixtures of gases*

As shown in Figure 1, intact pupae were placed in linear sequence in the depression of an elongate, semi-cylindrical tray of cellulose acetate and slipped into the

glass tube described under Method B by Harvey and Williams (1958). In experiments utilizing isolated hearts, the latter were pinned to a wax-coated tray and placed in the tube. The proximal end of the flow-tube was connected by a ground joint to a source of a constant flowing gas mixture; the distal end was connected by a ground joint to a length of rubber tubing which passed to a nearby chemical hood.

Specific mixtures of oxygen, nitrogen, carbon monoxide, and air were metered from Rotameters (Tri-Flat Variable-Area Flow Meters, Fisher and Porter, Co.). By selection of meters of appropriate capacity it was possible to utilize the several gases at rates of flow variable from 2 to 1000 cc. per minute, the error of each measurement not exceeding ten per cent. The several gas streams were combined and passed *via* rubber tubing to the experimental chamber, the total gas pressure being one atmosphere in all experiments of this type.

5. *Appraisal of heartbeat*

In experiments utilizing intact pupae, the average frequency and amplitude of the heartbeat were remarkably constant and predictable when computed for a series of individuals. The same was true to a somewhat lesser degree in experiments performed on isolated hearts in the flow-system described above. Therefore, in these types of preparations the average "heartbeat index" (Harvey and Williams, 1958) was used as an over-all expression of the average frequency and amplitude of the heartbeat. The frequency of heartbeat failed to show this same degree of regularity in the case of isolated hearts subjected to pressures greater than one atmosphere. Therefore, in experiments of this latter type, primary attention was centered on the amplitude of the beat rather than on frequency. Amplitude was scored on an arbitrary graded scale from 0 (no beat) to + + + (normal beat).

6. *Illumination*

By virtue of the transparent walls of the experimental chambers, the beating of the isolated heart was visible under the low magnification of the dissecting microscope. Incident illumination was utilized; namely, a focussed 15-watt lamp (Osram H3 6 volts) at a distance of approximately thirteen cm. from the preparation. In experiments pertaining to the light-reversibility of the inhibition, a second lamp (General Electric 1493, 30 watts) was also focussed on the same preparation. The lamps were equipped with one or more filters. An infrared filter (Corning No. 3962), was used routinely. Polarizing filters were used in studies of the intact animal (see above). And in experiments utilizing carbon monoxide mixtures, observations were performed in red light by the use of a Wratten filter (F filter No. 29).

7. *Reagents*

The experimental gases were obtained in compressed cylinders assaying as follows: "pre-purified nitrogen" (Airco), 99.998%; oxygen (Airco), 99.5%; anhydrous compressed air (New England Gas Products). The carbon monoxide was the Matheson product having the following composition: 96.8% carbon monoxide; 0.36% carbon dioxide; 0.97% hydrogen; 1% nitrogen; 0.8% saturated hydrocarbons; 1.19 mg. sulfur per liter. In order to minimize these several impurities, the gas was subdivided by sintered glass filters and bubbled through a succession

of two wash bottles containing 10 per cent potassium hydroxide; it then traversed a tube of anhydrous calcium chloride and passed to the flow-meters.

Results

1. *Effects of carbon monoxide on the heartbeat of pupae and adults*

The hearts of seven pupae and twelve adults of the Cecropia silkworm were isolated, moistened with a film of insect Ringer containing streptomycin sulfate and phenylthiourea, and placed in the transparent, high pressure chamber described above under Methods. The strength of the heartbeat was studied in red and in

Table I

Effects of carbon monoxide on the heartbeat of diapausing pupae and adult moths of Platysamia cecropia; light-reversibility of carbon monoxide inhibition

CO/O_2 Ratio	Oxygen tension (% atm.)	Amplitude of heartbeat			
		Diapausing pupae		Adult moths	
		Red light	White light	Red light	White light
100/1	5	+++	+++	++	+++
100/1	5	+++	+++	0	+++
100/1	5	++	+++	0	++
100/1	5			0	+++
100/1	5			+	+++
100/1	5			0	+++
100/1	5			0	+++
100/1	5			0	++
100/1	5			0	+++
80/1	5	+++	+++		
80/1	5	+++	+++		
80/1	5	+++	+++		
24/1	20	+++	+++	+	+++
24/1	20			+	+++
24/1	20			++	+++

white light in each of three CO/O_2 mixtures; namely, 100/1, 80/1, and 24/1, the oxygen tension in each case being recorded in Table I. Each preparation was observed for two hours.

The results are summarized in Table I. It will be observed that even the highest CO/O_2 ratios had little or no effect on the hearts of the diapausing pupae when the exposure was continued for two hours. By contrast, the adult hearts were strongly inhibited and this inhibition was fully or almost fully reversible in white light.

The light-reversibility in the case of adult hearts was especially dramatic when one region of heart was illuminated with white light and a closely adjacent region, with red light. The resumption of heartbeat in all cases was limited to the region

receiving the white light, the adjacent regions within the same heart remaining fully inhibited by carbon monoxide.

The experiments just considered for the Cecropia silkworm were repeated with similar results on adult hearts of the Polyphemus silkworm (*Telea polyphemus*).

The resistance of the pupal heart to high pressures of carbon monoxide was particularly impressive. However, it will be recalled that the heart at this stage possesses a substantial anaerobic metabolism (Harvey and Williams, 1958). Is it possible to account for the resistance to carbon monoxide on this basis? In order to test this possibility a series of four pupal hearts was isolated and exposed to a continuous flowing gas mixture containing 5% atm. oxygen and 95% atm. carbon monoxide (CO/O_2 ratio of 19/1). The carbon monoxide treatment was continued for 20 hours; *i.e.*, for a period greatly in excess of the anaerobic capacity of the pupal heart.

TABLE II

Effects on isolated hearts of prolonged exposure to a gas mixture containing 5 per cent oxygen in carbon monoxide (CO/O_2 ratio = 19/1)

Animal No.	Rate (beats/min.) and amplitude* of heartbeat								
	Air		Hours in CO/O_2 mixture						5 hours in air after 20 hours in CO/O_2 mixture
			2	$3\frac{3}{4}$	$5\frac{1}{2}$	7	$8\frac{1}{2}$	20	
1	14 (3)	16 (3)	0 (0)	10 (3)	10 (3)	13 (3)	3 (3)	13 (3)	9 (2)
2	6 (3)	9 (3)	7 (3)	9 (3)	11 (3)	3 (3)	3 (3)	2 (3)	0 (0)
3	11 (3)	12 (3)	5 (3)	6 (3)	8 (3)	8 (3)	9 (3)	6 (3)	6 (3)
4	20 (3)	23 (3)	17 (3)	9 (3)	12 (3)	9 (3)	13 (3)	7 (3)	3 (3)
Average heart-beat index**	13	15	7.2	8.5	10	8.2	7.0	7.0	3.5

* Amplitudes scored and recorded in parentheses after rate.

** See Harvey and Williams (1958) for calculation of average heartbeat index.

The results are summarized in Table II. When examined in red light, all hearts were still beating at the end of 20 hours of treatment; indeed, the average heartbeat index was as high at this time as after two hours of exposure to carbon monoxide. Moreover, the performance of the hearts was not improved when they were transferred to air at this time. Therefore, this experiment, like the preceding one, reveals little clear-cut evidence of sensitivity of the pupal heart to carbon monoxide.

2. *The adult heart: further studies on the photo-reversal of CO-inhibition*

A series of five adult hearts was isolated and exposed to a CO/O_2 ratio of 100/1, the oxygen tension being 5% atm. and the carbon monoxide tension 5 atm. Examination under red light confirmed the prompt cessation of heartbeat. In three preparations the effect was fully reversed by white light; in two preparations partially reversed.

The inhibited hearts were exposed, in turn, to a series of five emission lines from

a mercury lamp (General Electric, AH5, 200 watts). The various lines were isolated by an appropriate series of Corning filters. Table III records the various combinations of filters and the relative energies of the corresponding emission lines; the latter were determined by the use of a thermopile in conjunction with a U. S. Bureau of Standards source.

In the results recorded in Table III no compensation has been made for the dissimilar relative energies. Notwithstanding this fact, it is sufficiently clear that the 436 and 579 mμ lines were maximally effective in reversing the carbon monoxide effect. These wave-lengths are in good proximity to the 450 and 589 mμ absorption maxima reported for the cytochrome oxidase-carbon monoxide complex of rat heart (Melnick, 1942). Of special interest is the low effectiveness of light at

TABLE III

Effectiveness of mercury emission lines in reversing the CO-inhibition of adult Cecropia and Polyphemus hearts*

Species	Wave-length	White	365 mμ	405 mμ	436 mμ	546 mμ	579 mμ
	Relative energy**		0.69	0.49	1.00	4.27	1.71
	Corning filter combination		No. 7380 No. 5860	No. 3060 No. 4308 No. 5970	No. 3389 No. 5113	No. 3484 No. 5120 No. 4303	No. 3480 No. 4305
Cecropia	Amplitude of heartbeat	+++	0	+	+++	+	+++
Cecropia		++	0	0	0	0	+
Cecropia		++	0	+	+++	+	+
Polyphemus		+++	0	0	++	0	+
Polyphemus		+++	0	0	+++	0	+++

* 5% atm. O_2 plus 500% atm. CO (ratio $CO/O_2 = 100/1$).

** Multiplication by 701 ergs/cm.²/sec. converts to absolute energy.

546 mμ notwithstanding its high relative energy. This emission and those at 365 and 405 mμ fall in regions of minimal absorption reported for the cytochrome oxidase-carbon monoxide complex.

The action spectrum—rough as it is—leaves little doubt that the effect of carbon monoxide on the adult heart depends on its combination with cytochrome oxidase. Therefore, cytochrome oxidase may confidently be identified as the terminal oxidase of the adult heart. However, the results, up to this point, suggest that cytochrome oxidase may not play this same role in the pupal heart. For this reason the pupal heart was studied in further detail.

3. *Oxygen tension and the pupal heartbeat*

Physiological activities which depend on the function of cytochrome oxidase may be recognized, not only in terms of their light-reversible inhibition by carbon monoxide, but also by the fact that they proceed at normal rates at very low oxygen tensions. This results from the extraordinary affinity of cytochrome oxidase for oxygen. According to Winzler (1941), the oxygen consumption of yeast becomes

maximal when the partial pressure of oxygen is only 0.25 to 2.5 mm. Hg. (0.03% to 0.3% atm.)—an affinity for oxygen which far exceeds that of any other known oxidase.

Having failed by the use of carbon monoxide to identify the terminal oxidase in the pupal heart, we sought to clarify the matter by studying the effects of oxygen tension on the function of the pupal heart. For this purpose a series of ten intact diapausing pupae, selected for pale pigmentation, was placed dorsal-side-up in a glass tube. Flowing mixtures of oxygen and nitrogen were then prepared and circulated through the tube, as described above under Section 4 of Methods. The heartbeat was observed within the intact pupae by the use of polarized light.

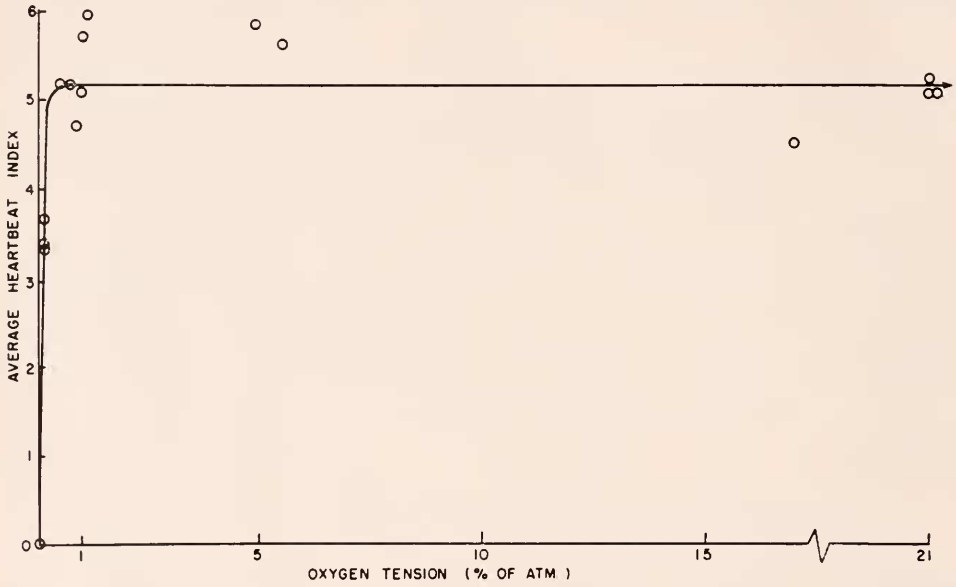


FIGURE 2. Effects of oxygen tension on the heartbeat of intact diapausing pupae of the Cecropia silkworm. The heartbeat is seen to be independent of oxygen at tensions at or above 0.5% atm. oxygen. In the determination of each datum, the exposure was continued for at least eight hours in order to compensate for the "anaerobic reserve."

In order to compensate for the anaerobic capacity of the pupal heart, the pupae were equilibrated with each gas mixture for eight hours prior to scoring the heartbeat. The same ten animals were used for the entire study, the tube being ventilated for eight hours with air before testing the next gas mixture.

The results are summarized and plotted in Figure 2 in terms of the average heartbeat index as a function of oxygen tension. It is extraordinary to observe that the heartbeat was independent of external oxygen tensions at or above 0.5% atm. Only when the pressure of oxygen was reduced below 0.5% atm. (4 mm. Hg) was there any detectable effect.

This result strongly argues that cytochrome oxidase is the terminal oxidase in the pupal heart, since no other oxidase is known to have the high affinity for oxygen implied in Figure 2.

4. *CO-inhibition of the pupal heartbeat at low oxygen tensions*

The finding that the hearts of intact diapausing pupae beat indefinitely at very low oxygen tensions paved the way for a more rigorous study of the effects of carbon monoxide than had been possible heretofore. Hearts could now be exposed to CO/O_2 ratios of 100/1 or higher. Moreover, the effects of carbon monoxide could be studied in the presence of very low oxygen tensions.

The following series of experiments had the objective of studying the heartbeat in the presence of varying pressures of carbon monoxide while holding the oxygen constant at a low pre-determined pressure. The constant oxygen pressures which were studied in turn were 0.18, 1.0, and 5.0% atm. The experimental set-up was essentially the same as that described under Section 3 above, except that the hearts were observed using polarized red light. Once again, ten intact diapausing pupae were used for the entire series of experiments.

TABLE IV

Effects of carbon monoxide at low oxygen tension on heartbeat of diapausing pupae

Ratio CO/O_2	5% atm. oxygen			1% atm. oxygen			0.18% oxygen		
	Average heartbeat index	Per cent inhibition	k(G)*	Average heartbeat index	Per cent inhibition	k(G)*	Average heartbeat index	Per cent inhibition	k(G)*
No CO	5.7			5.4			3.5		
3				5.4	0		1.6	53	2.4
10				3.5	35	19	0.8	76	3.2
18	5.6	2							
50				2.0	63	29	0.2	94	3.2
76	2.0	65	41						
99	1.3	78	28	0.9	83	20	0.1	97	3.0
555							0.0	100	
Average			34			23			2.9

* "Distribution constant" calculated from Warburg's partition equation (see text).

The first oxygen pressure to be studied in this manner was 0.18% atm. The pupae were equilibrated for eight hours with 0.18% atm. oxygen and 99.82% atm. of oxygen-free nitrogen. While holding the oxygen concentration constant, a certain percentage of carbon monoxide was then added to the flowing mixture of oxygen and nitrogen; namely, 0.54, 1.8, 9.0, 18.0, and 99.82% atm. carbon monoxide. The animals were equilibrated with each mixture for eight hours before recording the heartbeat. And before testing successive carbon monoxide mixtures, the system was ventilated for eight hours with 0.18% atm. oxygen and 99.82% atm. nitrogen.

The results are recorded in Table IV and the lower curve in Figure 3. It will be observed that in the absence of carbon monoxide the heartbeat index was already depressed by the low oxygen tension *per se*. As increasing percentages of carbon monoxide were added, one witnesses a further depression in heartbeat index attributable to carbon monoxide. Finally, at the low oxygen tension under consideration (0.18% atm.), the heartbeat is totally blocked at a CO/O_2 ratio of 555/1.

In like manner the experiment was repeated utilizing oxygen at the constant pressure of 1% atm. The results recorded in Table IV and Figure 3 show no depression attributable to the low oxygen pressure *per se*. However, as carbon monoxide is added, the heartbeat index begins to decline and continues to do so until one establishes a CO/O_2 ratio of 99/1—the highest ratio attainable at a total pressure of one atmosphere.

Finally, the experiment was repeated using oxygen at the constant pressure of 5% atm. A CO/O_2 ratio of 19/1 was the highest ratio attainable at one atmosphere total pressure. Therefore, the animals at this point were transferred to the transparent pressure chamber and the experiment continued at positive pressures up to

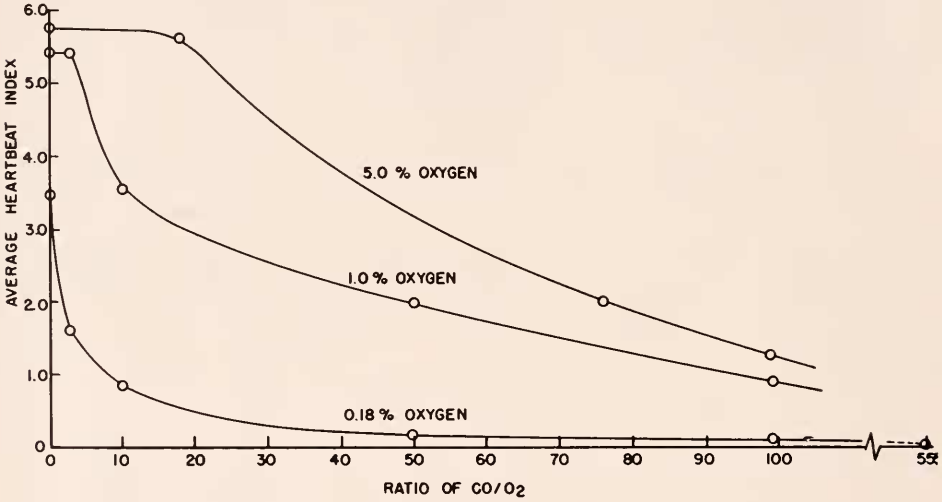


FIGURE 3. The effects of increasing concentrations of carbon monoxide on the heartbeat observed in intact diapausing Cecropia pupae. In the determination of each datum the experiment was continued for at least eight hours in order to compensate for the anaerobic reserve. The effects of carbon monoxide are dictated, not only by the CO/O_2 ratio, but also by the absolute tension of oxygen.

five atmospheres. The results summarized in Table IV and Figure 3 show no inhibition attributable to the low oxygen pressure *per se*. Moreover, the carbon monoxide inhibition now becomes apparent only at high CO/O_2 ratios; *i.e.*, in excess of 18/1.

5. *Photoreversal of the CO-inhibition of the pupal heart*

By the same technique described in Section 4, nine diapausing pupae were equilibrated for eight hours with a flowing mixture of 1% atm. oxygen and 99% atm. carbon monoxide (CO/O_2 ratio of 99/1). The hearts were then examined in polarized red light and the heartbeat index calculated. All hearts were strongly inhibited, and four of the nine were not beating. Each heart, in turn, was then illuminated with a pair of focussed beams of intense white light, and the effects scored over a period of five minutes. The results recorded in Table V reveal a marked reversal of the carbon monoxide inhibition by white light.

As a control for the preceding experiment, the same group of animals was equilibrated for eight hours with a flowing mixture of 1% atm. oxygen and 99% atm. nitrogen. The heartbeat index was then recorded in red light and white light, as just described. No inhibition was observed either in red or in white light (Table V).

TABLE V

Light reversibility of the CO-inhibition of pupal hearts (within intact animal)

Animal No.	Rate (beats/min.) and amplitude* of heartbeat			
	99% CO 1% O_2 Red light	99% CO 1% O_2 White light	99% N_2 1% O_2 Red light	99% N_2 1% O_2 White light
1	0 (0)	6 (3)	3 (3)	4 (3)
2	3 (1)	7 (3)	11 (3)	16 (3)
3	0 (0)	14 (3)	4 (3)	7 (3)
4	1 (1)	3 (3)	3 (3)	3 (3)
5	0 (0)	9 (3)	3 (3)	13 (3)
6	3 (2)	3 (3)	4 (3)	4 (3)
7	0 (0)	4 (3)	8 (3)	6 (3)
8	2 (1)	9 (3)	3 (3)	2 (3)
9	1 (2)	2 (3)	5 (3)	4 (3)
Average heartbeat index**	0.44	5.7	4.6	5.9

* Amplitudes scored and recorded in parentheses after rate.

** See Harvey and Williams (1958) for calculation of average heartbeat index.

6. *Lethal effects of carbon monoxide at low oxygen pressure*

In the experimental results described above, normal heartbeat was maintained in nitrogen containing 1% atm. oxygen. By contrast, the heart was strongly inhibited in carbon monoxide containing 1% atm. oxygen. These results lead to the prediction that carbon monoxide should be a lethal agent for diapausing pupae when administered in combination with low oxygen pressure.

Five diapausing pupae were placed in each of two 3.5-liter Lucite pressure chambers along with a small dish of 10 per cent potassium hydroxide to absorb carbon dioxide. One chamber was flushed daily with ten volumes of a gas mixture containing 1% atm. oxygen in nitrogen; the other, with a mixture of 1% atm. oxygen in carbon monoxide. The experiment was continued for 14 days. The chambers were then opened and the animals examined. All of the control animals in nitrogen were lively and normal; all the experimental animals in carbon monoxide were flaccid and dead.

DISCUSSION

1. *The heartbeat of the adult moth*

The heartbeat of the adult moth is strongly inhibited by cyanide in that HCN, in a concentration of less than 10^{-5} *M*, inhibits the heartbeat by 50 per cent (Harvey

and Williams, 1958). This finding is strong evidence that the heartbeat in the adult moth depends on the function of cytochrome oxidase.

In the present study the isolated adult heart was inhibited when the ratio of carbon monoxide to oxygen was 24/1 in the surrounding gas phase. This inhibition was promptly reversed by light. Moreover, the effectiveness of the light coincides with the absorption maxima of the cytochrome oxidase-CO complex. Therefore, the terminal oxidase of the adult heart may confidently be identified as cytochrome oxidase. This conclusion is consistent with the finding that the adult heart contains a high concentration of the several cytochromes including cytochrome oxidase (Shappirio and Williams, 1957a, 1957b).

2. The heartbeat of the diapausing pupa

As described in Sections 1 and 4 of the Results, it is easy to get the impression that the pupal heart differs from the adult heart in being insensitive to carbon monoxide. Even when the ratio of carbon monoxide to oxygen is raised as high as 19/1, the pupal heart continues to beat normally, provided that at least 5% atm. oxygen is present.

In the analogous case of the apparent insensitivity of the pupal heart to cyanide (Harvey and Williams, 1958), a satisfactory explanation was found to be the anaerobic capacity of the heart at this particular stage. However, this same explanation does not suffice to explain the insensitivity of the pupal heart to carbon monoxide. Thus, as we have seen (Table II), the pupal heart beats indefinitely in mixtures of carbon monoxide and oxygen (19/1), whereas it comes to a standstill after several hours in oxygen-free nitrogen. Therefore, on the basis of results of this type, one might be persuaded that the pupal heart fails to make use of CO-sensitive cytochrome oxidase, notwithstanding the presence in the pupal heart of a substantial titer of this same enzyme.

The present study makes clear that this conclusion is erroneous. It is our present purpose to show how the relatively high concentration of cytochrome oxidase in the pupal heart serves to camouflage the true sensitivity of the pupal heart to carbon monoxide.

3. Pupal heartbeat at low oxygen tensions

The resistance of the pupal heart to carbon monoxide can be accounted for most easily by first considering the insensitivity of the same heart to low oxygen tensions. The two phenomena, as we shall see, have a very close connection.

As illustrated in Figure 2, the heart of the diapausing pupa continues to beat normally for an indefinite period when the intact pupa is exposed to oxygen tensions as low as 0.5% atm. In experiments which are sufficiently prolonged to compensate for the anaerobic reserve of the pupal heart, one finds that the heartbeat is blocked in the total absence of oxygen and significantly depressed at external tensions lower than 0.5% atm. This implies that, after the exhaustion of its anaerobic reserve, the pupal heart is "micro-aerophilic." Oxygen is required, but a very low tension suffices. The fact that an external tension as low as 0.5% atm. sustains the normal heartbeat is especially impressive when one recalls that a tension of 0.5% atm. oxygen outside the insect corresponds to a still lower tension in the heart muscle itself.

In our opinion the micro-aerophilic character of the pupal heart can be accounted for in terms of the presence in the pupal heart of a substantial titer of cytochrome oxidase in conjunction with only a trace of its substrate *c*. Consider the situation in Figure 4A descriptive of the circumstances in the heart of the diapausing pupa. Here the trace of cytochrome *c* is diagrammed as passing electrons to the oxidized heme (+ + +) of cytochrome oxidase. The latter is present in the diapausing heart in great excess over cytochrome *c*. Provided that the trace of *c* has free access to the pool of oxidase, a steady-state will be established in which the reduction of oxidase is more than counterbalanced by the oxidation of oxidase by molecular oxygen. Therefore, the vast majority of cytochrome oxidase exists in the oxidized state. As diagrammed in Figure 5A, one can lower the oxygen tension, thereby establishing a new steady-state in which half of the oxidase is in the oxidized form and half in the reduced form. The respiration, as signalled by the rate of water formation, remains unimpaired.

The presence of the great excess of cytochrome oxidase has a further influence on the kinetics of this steady state. At very low oxygen tensions, most of the oxidase will be present in the reduced (+ +) state. This increases the concentration of reduced oxidase, the target of molecular oxygen, and permits a still lower oxygen tension to suffice. The excess oxidase acts, in effect, as a "buffer" against very low oxygen tensions. It is important to note that under all these circumstances the rate of electron transmission and of oxygen consumption (represented by the block marked H_2O in the diagrams) remain constant and independent of oxygen tension.

The set of circumstances, diagrammed in Figure 6A, considers the situation when the oxygen tension is so low as to limit the respiration. Almost all of the oxidase is now reduced. Finally, in a completely anaerobic situation, all the oxidase becomes reduced and the respiration is totally blocked at this point.

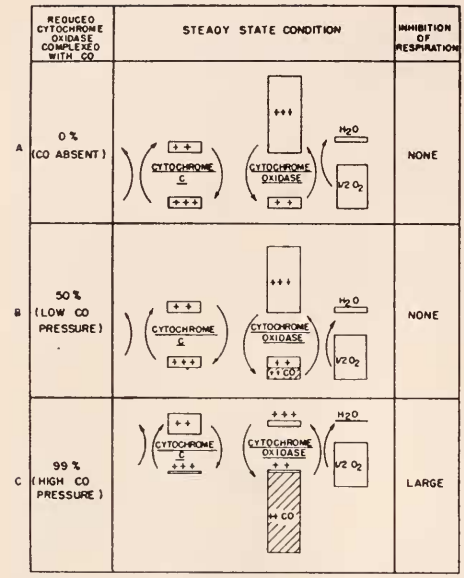
For these several reasons we can understand the reason why the pupal heart continues to beat normally in the presence of oxygen tensions as low as 0.5% atm. The explanation is found to lie in the disproportionately high concentration of cytochrome oxidase with respect to cytochrome *c*—the precise combination that characterizes the heart of the diapausing pupa (Shappirio and Williams, 1957a, 1957b). The surplus oxidase is "money in the bank" which can be drawn upon for transactions at low oxygen pressures.

4. *A theoretical consideration of CO-inhibition*

As Goddard (1947) points out, a mathematical treatment of the kinetics of the CO-inhibition of cytochrome oxidase requires so many simplifying assumptions that the formulation is scarcely descriptive of any real experiment. Indeed, we are unable to find in a search of the literature any satisfactory non-mathematical treatment. Consequently, we have been forced to develop a semi-diagrammatic presentation of the facts. Though descriptive of the experiments on the silkworm heart, we believe that the formulation will be pertinent to analogous studies.

A. *CO-inhibition at normal oxygen pressures*

The crux of the matter is that carbon monoxide combines only with the reduced form of cytochrome oxidase, the CO/O_2 ratio dictating the percentage of reduced oxidase which is complexed and inactivated. By virtue of the low concentration of



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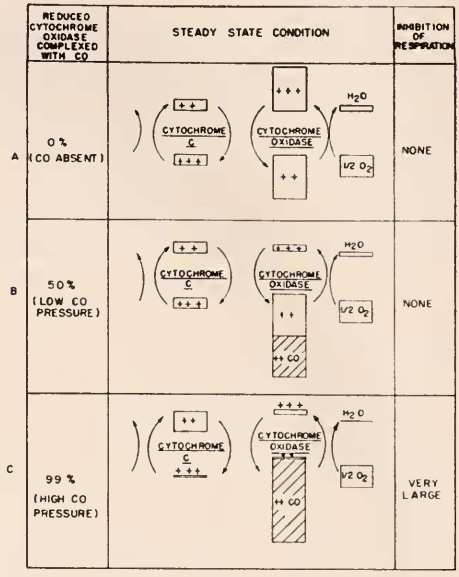

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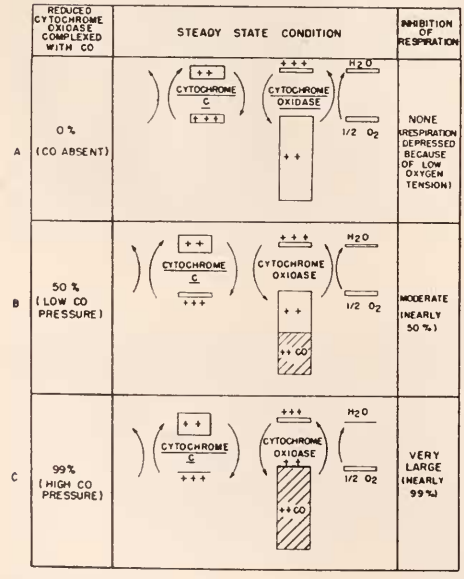

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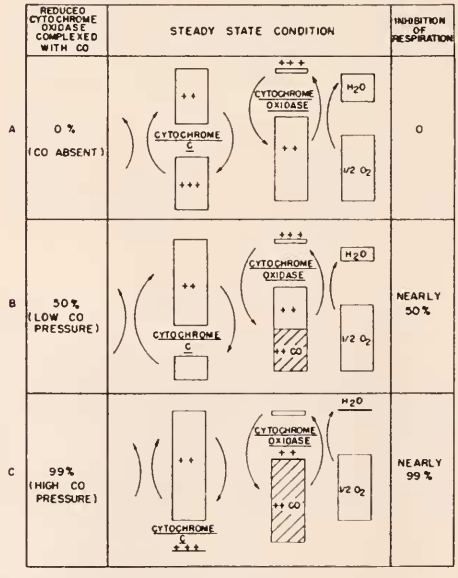

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FIGURE 4. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the steady-state condition of the electron transport system when the oxygen tension is not limiting respiration (5% atm. oxygen or above).

cytochrome *c* a correspondingly low concentration of reduced oxidase exists in the normal pupal heart at ordinary oxygen tensions (Fig. 4A). Under this circumstance carbon monoxide finds a limited target within the diapausing heart. The addition of sufficient carbon monoxide to complex, say, 50 per cent of the reduced oxidase transiently slows the rate of oxidation of reduced cytochrome oxidase. This causes additional oxidase to accumulate in the reduced form until the amount of reduced oxidase is twice that present in the absence of carbon monoxide (Fig. 4B). Although 50 per cent continues to be complexed by carbon monoxide, in the new steady-state the amount of uncomplexed reduced oxidase becomes the same as it had been in the total absence of carbon monoxide. Consequently, the respiration is uninhibited.

At very high pressures of carbon monoxide sufficient to complex, say, 99 per cent of reduced oxidase, the reserve of oxidase becomes limiting and the system can no longer undergo the necessary degree of internal compensation (Fig. 4C). Therefore, the rates of electron transfer, oxygen consumption, and water formation are slowed down.

B. *CO-inhibition at low oxygen pressures*

As we have just seen, the concentration of reduced cytochrome oxidase and the sensitivity to carbon monoxide can be enhanced experimentally by lowering the oxygen tension. Let us consider the hypothetical case where the oxygen pressure is lowered until 50 per cent of the oxidase is in the reduced form (Fig. 5A). The rate of oxygen consumption and water formation remains the same as at higher oxygen pressures.

If one now adds enough carbon monoxide to complex 50 per cent of the reduced oxidase, a new steady-state results in which nearly all the oxidase shifts to the reduced condition (Fig. 5B). Whether the respiration will be inhibited will be determined by whether sufficient oxidase is present to supply the necessary degree of compensation. In the case considered in Figure 5B, this condition is fulfilled and the rate of water formation is diagrammed as uninhibited. However, as shown in Figure 5C, the compensatory mechanism breaks down if the pressure of carbon monoxide is further increased. A strong inhibition of respiration and of water formation is then observed.

C. *CO-inhibition at very low oxygen pressures*

Attention is finally directed to the set of circumstances diagrammed in Figure 6. The oxygen pressure at the outset is reduced to a very low level (0.18% atm.) so that the respiration is already inhibited and most of the cytochrome oxidase is present in the reduced form (Fig. 6A). The reserves of oxidized

FIGURE 5. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the electron transport system when the oxygen tension is low but not limiting respiration (1% atm. oxygen).

FIGURE 6. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the electron transport system when the oxygen tension is limiting respiration (0.18% atm. oxygen).

FIGURE 7. CO-inhibition of the respiration of developing adults of the Cecropia silkworm. Diagram of the steady-state condition of the electron transport system when the oxygen tension is not limiting the respiration. The resynthesis of cytochrome *c* enhances the metabolism and the sensitivity to carbon monoxide.

oxidase have already been exhausted and the system is immediately sensitive to low pressures of carbon monoxide (Figs. 6B and 6C).

5. *Carbon monoxide and the pupal heart*

Under experimental conditions the pupal heart is found to respond to carbon monoxide in accordance with theory. In Figure 8 the experimental results of

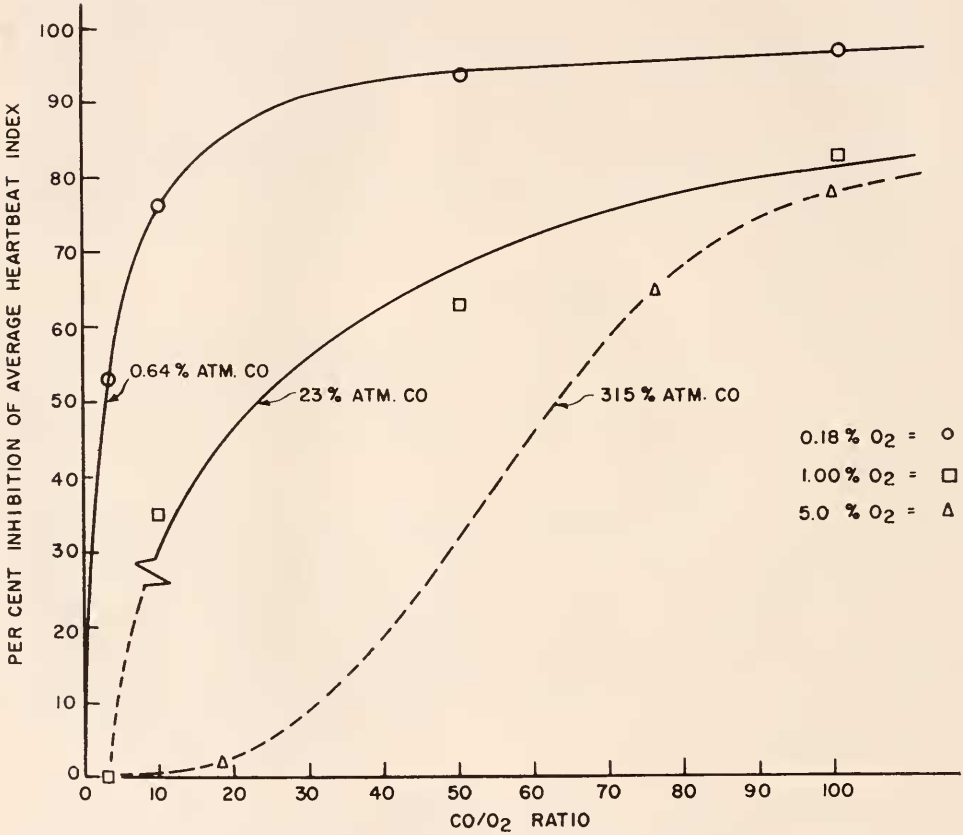

FIGURE 8. Per cent inhibition of the heartbeat of diapausing Cecropia pupae as a function of the CO/O_2 ratio. The inhibition is determined, not only by the CO/O_2 ratio, but also by the absolute tension of oxygen. The uppermost curve has been drawn in accordance with the Warburg equation.

Table IV and Figure 3 have been brought together and plotted on a scale in which the per cent inhibition of heartbeat is considered as a function of the CO/O_2 ratio at each of three oxygen pressures.

At the very low oxygen pressure of 0.18% atm., the heart is inhibited by any finite pressure of carbon monoxide and 50 per cent inhibited by carbon monoxide at a pressure of only 0.64% atm. (5 mm. Hg). When the oxygen pressure is raised to 1% atm., then the carbon monoxide pressure must be increased to 23%

atm. to achieve 50 per cent inhibition. Finally, at the still higher oxygen pressure of 5% atm., 50 per cent inhibition is brought about only by a very high pressure of carbon monoxide (315% atm.).

6. The critical effects of cytochrome c

The analysis, up to this point, has focussed attention on the oxidation of cytochrome oxidase by molecular oxygen. In a given system at low oxygen pressures, the pressure of oxygen dictates the partition of oxidase between oxidized and reduced states; this partition, in turn, is found to condition the sensitivity to carbon monoxide. An examination of Figure 7 will make clear that this same partition can be influenced at any specific oxygen pressure by varying the rate of reduction of cytochrome oxidase. This is achieved by increasing the concentration of reduced cytochrome *c*.

It is precisely this circumstance which supervenes at the initiation of adult development. As Shappirio and Williams (1957a, 1957b) have shown, a rapid synthesis of cytochrome *c* occurs at this time. The metabolism is enhanced and now shows a definite sensitivity to carbon monoxide even at high oxygen pressures (Schneiderman and Williams, 1954a). This result is intelligible in terms of the discussion set forth above.

Shappirio and Williams (1957b) have been able to duplicate this same phenomenon *in vitro* by the addition of extrinsic cytochrome *c* to washed homogenates of diapausing pupal tissues (using DPNH as substrate). The metabolism of the homogenate increases markedly and shows a substantial sensitivity to inhibitors of cytochrome oxidase.

7. Warburg's equation for CO-inhibition

In the hypothetical situation where all the cytochrome oxidase is in the reduced condition, then, as Warburg (1927) points out, one can formulate a simple stoichiometric relation between the inhibition of respiration and the CO/O_2 ratio.

$$\frac{\text{Respiration uninhibited by CO}}{\text{Respiration inhibited by CO}} = K\,\frac{O_2}{CO}$$

In practice it is difficult or impossible to establish a steady-state in which all the oxidase is reduced. Biochemists have routinely attempted to satisfy this requirement by the addition to *in vitro* systems of a large excess of substrates and cytochrome *c*.

The present investigation directs attention to a far simpler solution of the problem which is applicable, not only to *in vitro* systems, but also to intact organisms. This technique, as we have seen, is to favor the reduction of oxidase by working at very low oxygen pressures. Under this circumstance the quantitative aspects of the carbon monoxide inhibition are in precise agreement with Warburg's formulation. This fact is evident in Figure 8 where the uppermost curve is a theoretical curve constructed according to the Warburg equation, the constant K being 3. This low value implies that carbon monoxide has a higher affinity for reduced cytochrome oxidase than is customarily assumed.

8. *The terminal oxidase during metamorphosis*

We are persuaded by the argument outlined above that the heartbeat of the diapausing pupa is sustained by the CO-sensitive cytochrome oxidase. Confirmation of this view is found in the demonstration that the CO-inhibition of the pupal heartbeat is promptly reversed by light (Table V). The reason that the pupal heart appears to be insensitive to carbon monoxide is that the true sensitivity is camouflaged by the great excess of cytochrome oxidase that is present. In our opinion many additional instances of so-called CO-insensitive respiration reported in the literature will be found to have a similar basis.

In the earlier studies we have interpreted the CO-resistance of the diapausing pupa to signal the presence of a CO-insensitive oxidase. However, it is worth recalling that the non-muscular tissues of the diapausing pupa also contain cytochrome oxidase in great excess over cytochrome *c* (Shappirio and Williams, 1957a and b). Therefore, if cytochrome oxidase can give the false impression of CO-insensitivity in the heart, there is no *a priori* reason why it cannot do so in the non-muscular tissues. In this connection it is of interest that the pupa as a whole is killed by exposure to carbon monoxide at low oxygen pressures; *i.e.,* under conditions where the excess of cytochrome oxidase is eliminated from the field of action (Section 6 of Results).

The present study of the heart does not permit a clear decision as to the role of cytochrome oxidase in the pupa as a whole. Most fortunately, however, this particular matter has simultaneously been studied in an independent investigation by Kurland and Schneiderman, and will be considered in detail in a forthcoming publication.

Summary

1. The heartbeat of the adult Cecropia moth is inhibited by suitable pressures of carbon monoxide, and this inhibition is reversed by light.

2. The wave-lengths which are maximally effective in reversing the CO-inhibition are in good agreement with the absorption maxima of the CO-cytochrome oxidase complex.

3. Therefore, the terminal oxidase of the adult heart may be identified as cytochrome oxidase.

4. The situation is much more complex in the case of the diapausing pupa. The latter shows a considerable capacity for anaerobic metabolism, and its heart can beat for several hours in the total absence of oxygen. Moreover, normal heartbeat continues indefinitely in the presence of oxygen pressures as low as 0.5% atm.

5. After exhaustion of the "anaerobic reserve," the pupal heart may be said to be "micro-aerophilic." Oxygen is required but a remarkably low tension suffices.

6. At ordinary oxygen pressures such as that in air, it is difficult or impossible to demonstrate any sensitivity of the pupal heart to carbon monoxide. However, when the oxygen tension is decreased to very low levels, the heartbeat shows a clear sensitivity to carbon monoxide. Thus, at the low oxygen tension of 0.18% atm., the pupal heartbeat is inhibited 50 per cent by carbon monoxide at a pressure of only 5 mm. Hg.

7. Under these circumstances, the inhibition of the pupal heartbeat is light-reversible.

8. The actions of oxygen and carbon monoxide are considered in detail. The terminal oxidase of the pupal heart is found to be cytochrome oxidase.

9. The resistance of the pupal heart to carbon monoxide and to low oxygen pressures can be accounted for in terms of the presence in the pupal heart of a great excess of cytochrome oxidase relative to cytochrome *c*.

LITERATURE CITED

Ephrussi, B., and G. W. Beadle, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.*, **52**: 218–225.

Goddard, D. R., 1947. Respiratory enzymes. *In:* Physical Chemistry of Cells and Tissues, (R. Höber, editor), London. J. and A. Churchill, Ltd.: 397–420.

Harvey, W. R., and C. M. Williams, 1958. Physiology of insect diapause. XI. Cyanide-sensitivity of the heartbeat of the Cecropia silkworm, with special reference to the anaerobic capacity of the heart. *Biol. Bull.*, **114**: 23–35.

Hill, R., and E. F. Hartree, 1953. Hematin compounds in plants. *Ann. Rev. Plant Physiol.*, **4**: 115–150.

Melnick, J. L., 1942. The photochemical spectrum of cytochrome oxidase. *J. Biol. Chem.*, **146**: 385–390.

Schneiderman, H. A., and C. M. Williams, 1954a. The physiology of insect diapause. VIII. Qualitative changes in the metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.*, **106**: 210–229.

Schneiderman, H. A., and C. M. Williams, 1954b. The physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.*, **106**: 238–252.

Schneiderman, H. A., and N. Feder, 1954. A respirometer for metabolic studies at high gaseous pressures. *Biol. Bull.*, **106**: 230–237.

Shappirio, D. G., and C. M. Williams, 1957a. The cytochrome system of the Cecropia silkworm. I. Spectroscopic studies of individual tissues. *Proc. Roy. Soc. London, Ser. B*, **147**: 218–232.

Shappirio, D. G., and C. M. Williams, 1957b. The cytochrome system of the Cecropia silkworm. II. Spectrophotometric studies of oxidative enzyme systems in the wing epithelium. *Proc. Roy. Soc. London, Ser. B*, **147**: 233–246.

Warburg, O., 1927. Über die Wirkung von Kohlenoxyd und Stickoxyd auf Atmung und Gärung. *Biochem. Zeitschr.*, **189**: 354–380.

Warburg, O., 1949. Heavy metal prosthetic groups and enzyme action. Oxford at the Clarendon Press. pp. 230.

Williams, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.* **90**: 234–243.

Winzler, R. J., 1941. The respiration of bakers' yeast at low oxygen tension. *J. Cell. Comp. Physiol.*, **17**: 263–276.